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(54) Title: POLYPEPTIDES HOMOLOGOUS TO VEGF AND BMP1

(57) Abstract

The present invention involves the identification and preparation of vascular endothelial growth factor–E (VEGF–E). VEGF–E is a novel polypeptide related to vascular endothelial growth factor (VEGF) and bone morphogenetic protein 1. VEGF–E has homology to VEGF including conservation of the amino acids required for activity of VEGF. VEGF–E can be useful in wound repair, as well as in the generation and regeneration of tissue.

POLYPEPTIDES HOMOLOGOUS TO VEGF AND BMP1

Field of the Invention

The present invention is directed to polypeptides related to vascular endothelial cell growth factor (hereinafter sometimes referred to as VEGF) and bone morphogenetic protein 1 (hereinafter sometimes referred to as BMP1), termed herein as VEGF-E polypeptides, nucleic acids encoding therefor, methods for preparing VEGF-E, and methods, compositions, and assays utilizing VEGF-E.

Background of the Invention

Various naturally occurring polypeptides reportedly induce the proliferation of endothelial cells. Among those polypeptides are the basic and acidic fibroblast growth factors (FGF) (Burgess and Maciag, Annual Rev. Biochem., 58: 575 (1989)), platelet-derived endothelial cell growth factor (PD-ECGF) (Ishikawa *et al.*, Nature, 338: 557 (1989)), and vascular endothelial growth factor (VEGF). Leung *et al.*, Science, 246: 1306 (1989); Ferrara and Henzel, Biochem. Biophys. Res. Commun., 161: 851 (1989); Tischer *et al.*, Biochem. Biophys. Res. Commun., 165: 1198 (1989); EP 471,754B granted July 31, 1996.

The heparin-binding endothelial cell-growth factor, VEGF, was identified and purified from media conditioned by bovine pituitary follicular or folliculo-stellate cells several years ago. See Ferrara *et al.*, Biophys. Res. Comm., 161: 851 (1989). Media conditioned by cells transfected with the human VEGF (hVEGF) cDNA promoted the proliferation of capillary endothelial cells, whereas control cells did not. Leung *et al.*, Science, 246: 1306 (1989). VEGF is a naturally occurring compound that is produced in follicular or folliculo-stellate cells (FC), a morphologically well-

and vascular injuries resulting from trauma such as subcutaneous wounds. Being a vascular (artery and venus) endothelial cell growth factor, VEGF restores cells that are damaged, a process referred to as vasculogenesis, and stimulates the formulation of new vessels, a process referred to as angiogenesis.

VEGF would also find use in the restoration of vasculature after a myocardial infarct, as well as other uses that can be deduced. In this regard, inhibitors of VEGF are sometimes desirable, particularly to mitigate processes such as angiogenesis and vasculogenesis in cancerous cells.

It is now well established that angiogenesis, which involves the formation of new blood vessels from preexisting endothelium, is implicated in the pathogenesis of a variety of disorders. These include solid tumors and metastasis, atherosclerosis, retrolental fibroplasia, hemangiomas, chronic inflammation, intraocular neovascular syndromes such as proliferative retinopathies, *e.g.*, diabetic retinopathy, age-related macular degeneration (AMD), neovascular glaucoma, immune rejection of transplanted corneal tissue and other tissues, rheumatoid arthritis, and psoriasis. Folkman *et al.*, J. Biol. Chem., 267: 10931-10934 (1992); Klagsbrun *et al.*, Annu. Rev. Physiol., 53: 217-239 (1991); and Garner A, "Vascular diseases", In: Pathobiology of Ocular Disease. A Dynamic Approach, Garner A, Klintworth GK, Eds., 2nd Edition (Marcel Dekker, NY, 1994), pp 1625-1710.

In the case of tumor growth, angiogenesis appears to be crucial for the transition from hyperplasia to neoplasia, and for providing nourishment to the growing solid tumor. Folkman *et al.*, Nature, 339: 58 (1989). The neovascularization allows the tumor cells to acquire a growth advantage and proliferative autonomy compared to the normal cells. Accordingly, a correlation has been observed between density of microvessels in tumor sections and patient survival in breast cancer as well as in several other tumors. Weidner *et al.*, N Engl J Med, 324: 1-6 (1991); Horak *et al.*, Lancet, 340: 1120-1124 (1992); Macchiarini *et al.*, Lancet, 340: 145-146 (1992).

The search for positive regulators of angiogenesis has yielded many candidates, including aFGF, bFGF, TGF-α, TGF-β, HGF, TNF-α, angiogenin, IL-8, etc. Folkman *et al.*, J.B.C., *supra*, and Klagsbrun

antibodies are described, for example, in EP 817,648 published January 14, 1998 and in PCT/US 98/06724 filed April 3, 1998.

Regarding the bone morphogenetic protein family, members of this family have been reported as being involved in the differentiation of cartilage and the promotion of vascularization and osteoinduction in preformed hydroxyapatite. Zou, *et al.*, Genes Dev. (U.S.), 11(17):2191 (1997); Levine, *et al.*, Ann. Plast. Surg., 39(2):158 (1997). A number of related bone morphogenetic proteins have been identified, all members of the bone morphogenetic protein (BMP) family. Bone morphogenetic native and mutant proteins, nucleic acids encoding them, related compounds including receptors, host cells, and uses are further described in at least: U.S. Patent Nos. 5,670,338; 5,454,419; 5,661,007; 5,637,480; 5,631,142; 5,166,058; 5,620,867; 5,543,394; 4,877,864; 5,013,649; 5,106,748; and 5,399,677. Of particular interest are proteins having homology with bone morphogenetic protein 1, a procollagen C-proteinase that plays key roles in regulating matrix deposition. In view of the role of vascular endothelial cell growth and angiogenesis in many diseases and disorders, it is desirable to have a means of reducing or inhibiting one or more of the biological effects causing these processes. It is also desirable to have a means of assaying for the presence of pathogenic polypeptides in normal and diseased conditions, and especially cancer. Further, in a specific aspect, as there is no generally applicable therapy for the treatment of cardiac hypertrophy, the identification of factors that can prevent or reduce cardiac myocyte hypertrophy is of primary importance in the development of new therapeutic strategies to inhibit pathophysiological cardiac growth. While there are several treatment modalities for various cardiovascular and oncologic disorders, there is still a need for additional therapeutic approaches.

The present invention is predicated upon research intended to identify novel polypeptides which are related to VEGF and the BMP family, and in particular, polypeptides which have a role in the survival, proliferation, and/or differentiation of cells. While the novel polypeptides are not expected to have biological activity identical to the known polypeptides to which they have homology, the known polypeptide biological activities can be used to determine the

In another aspect of the invention is provided a composition comprising the VEGF-E polypeptide in admixture with a carrier. In a preferred aspect, the composition comprises a therapeutically effective amount of the polypeptide, wherein the carrier is a pharmaceutically acceptable carrier. Also preferred is where the composition further comprises a cardiovascular, endothelial, or angiogenic agent.

In a still further embodiment, the invention provides a method for preparing the composition for the treatment of a cardiovascular or endothelial disorder comprising admixing a therapeutically effective amount of the VEGF-E polypeptide with the carrier.

In another embodiment, the invention provides a pharmaceutical product comprising:

(a) the composition described above;

(b) a container containing said composition; and

(c) a label affixed to said container, or a package insert included in said pharmaceutical product referring to the use of said VEGF-E polypeptide in the treatment of a cardiovascular or endothelial disorder.

In yet another embodiment, the invention provides a method for diagnosing a disease or a susceptibility to a disease related to a mutation in a nucleic acid sequence encoding VEGF-E comprising:

(a) isolating a nucleic acid sequence encoding VEGF-E from a sample derived from a host; and

(b) determining a mutation in the nucleic acid sequence encoding VEGF-E.

In a still further embodiment, the invention provides a method of diagnosing cardiovascular and endothelial disorders in a mammal comprising detecting the level of expression of a gene encoding a VEGF-E polypeptide (a) in a test sample of tissue cells obtained from the mammal, and (b) in a control sample of known normal tissue cells of the same cell type, wherein a higher or lower expression level in the test sample indicates the presence of a cardiovascular or endothelial dysfunction in the mammal from which the test tissue cells were obtained.

In a further embodiment, the invention provides a method for treating a cardiovascular or endothelial disorder in a mammal comprising administering to the mammal an effective amount of a

to the mammal an effective amount of an antagonist to a VEGF-E polypeptide. In a preferred embodiment, the angiogenic disorder is cancer or age-related macular degeneration. In another preferred embodiment, the mammal is human. In a further preferred aspect, an effective amount of an angiostatic agent is administered in conjunction with the antagonist.

In other aspects, the invention provides an isolated antibody that binds a VEGF-E polypeptide. Preferably, this antibody is a monoclonal antibody.

In a further aspect, the invention provides a method for inhibiting angiogenesis induced by VEGF-E polypeptide in a mammal comprising administering a therapeutically effective amount of the antibody to the mammal, where preferably the mammal is a human. Also, the mammal preferably has a tumor or a retinal disorder. In another preferred aspect, the mammal has cancer and the antibody is administered in combination with a chemotherapeutic agent, a growth inhibitory agent, or a cytotoxic agent.

In another preferred embodiment, the invention provides a method for determining the presence of a VEGF-E polypeptide comprising exposing a cell suspected of containing the VEGF-E polypeptide to the antibody and determining binding of said antibody to said cell.

In yet another preferred aspect, the invention supplies a method of diagnosing cardiovascular, endothelial, or angiogenic disorders in a mammal comprising (a) contacting the antibody with a test sample of tissue cells obtained from the mammal, and (b) detecting the formation of a complex between the anti-VEGF-E antibody and the VEGF-E polypeptide in the test sample.

In still further aspects, the invention provides a cancer diagnostic kit comprising the antibody and a carrier in suitable packaging. Preferably, the kit further comprises instructions for using said antibody to detect the VEGF-E polypeptide.

In yet another embodiment, the invention provides an article of manufacture, comprising:

a container;

a label on the container; and

a composition comprising an anti-VEGF-E antibody contained within the container; wherein the label on the container indicates

Detailed Description of the Invention

I. Definitions

As used herein, "vascular endothelial cell growth factor-E," or "VEGF-E," refers to a mammalian growth factor as described herein, including the human amino acid sequence of Figure 2, a sequence which has homology to VEGF and bone morphogenetic protein 1 and which includes complete conservation of all VEGF cysteine residues, which have been shown to be required for biological activity of VEGF. VEGF-E expression includes expression in human fetal bone, thymus, and the gastrointestinal tract, as well as in fetal testis, lung, and lymph nodes, and in other tissues as shown in the examples below. The biological activity of native VEGF-E is shared by any analogue or variant thereof that promotes selective growth and/or survival of umbilical vein endothelial cells, induces proliferation of pluripotent fibroblast cells, induces immediate early gene c-fos in human endothelial cell lines, causes myocyte hypertrophy in cardiac cells, inhibits VEGF-stimulated proliferation of adrenal cortical capillary endothelial cells, or which possesses an immune epitope that is immunologically cross-reactive with an antibody raised against at least one epitope of the corresponding native VEGF-E. The human VEGF-E herein is active on rat and mouse cells, indicating conservation across species. Moreover, the VEGF-E herein is expressed at the growth plate region and has been shown to embrace fetal myocytes.

As used herein, "vascular endothelial cell growth factor," or "VEGF," refers to a mammalian growth factor as defined in U.S. Patent 5,332,671. The biological activity of native VEGF is shared by any analogue or variant thereof that promotes selective growth of vascular endothelial cells but not of bovine corneal endothelial cells, lens epithelial cells, adrenal cortex cells, BHK-21 fibroblasts, or keratinocytes, or that possesses an immune epitope that is immunologically cross-reactive with an antibody raised against at least one epitope of the corresponding native VEGF.

The terms "VEGF-E polypeptide" and "VEGF-E" when used herein encompass native-sequence VEGF-E polypeptide and VEGF-E polypeptide variants (which are further defined herein). The VEGF-E polypeptides may be isolated from a variety of sources, such as from

Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

"Percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotides in a candidate sequence that are identical with the sequence shown in Figure 1 (SEQ ID NO:1), respectively, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the VEGF-E polypeptide natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated" VEGF-E polypeptide-encoding nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the VEGF-E polypeptide-encoding nucleic acid. An isolated VEGF-E polypeptide-

is due either to an increase in the mass of the individual cells (true hypertrophy), or to an increase in the number of cells making up the tissue (hyperplasia), or both. Certain organs, such as the heart, lose the ability to divide shortly after birth. Accordingly, "cardiac hypertrophy" is defined as an increase in mass of the heart, which, in adults, is characterized by an increase in myocyte cell size and contractile protein content without concomitant cell division. The character of the stress responsible for inciting the hypertrophy, (*e.g.*, increased preload, increased afterload, loss of myocytes, as in myocardial infarction, or primary depression of contractility), appears to play a critical role in determining the nature of the response. The early stage of cardiac hypertrophy is usually characterized morphologically by increases in the size of microfibrils and mitochondria, as well as by enlargement of mitochondria and nuclei. At this stage, while muscle cells are larger than normal, cellular organization is largely preserved. At a more advanced stage of cardiac hypertrophy, there are preferential increases in the size or number of specific organelles, such as mitochondria, and new contractile elements are added in localized areas of the cells, in an irregular manner. Cells subjected to long-standing hypertrophy show more obvious disruptions in cellular organization, including markedly enlarged nuclei with highly lobulated membranes, which displace adjacent myofibrils and cause breakdown of normal Z-band registration. The phrase "cardiac hypertrophy" is used to include all stages of the progression of this condition, characterized by various degrees of structural damage of the heart muscle, regardless of the underlying cardiac disorder. Hence, the term also includes physiological conditions instrumental in the development of cardiac hypertrophy, such as elevated blood pressure, aortic stenosis, or myocardial infarction.

"Heart failure" refers to an abnormality of cardiac function where the heart does not pump blood at the rate needed for the requirements of metabolizing tissues. The heart failure can be caused by a number of factors, including ischemic, congenital, rheumatic, or idiopathic forms.

"Congestive heart failure" or "CHF" is a progressive pathologic state where the heart is increasingly unable to supply adequate

diastolic function. Inouye *et al.*, Am. J. Cardiol., 53: 1583-7 (1984).

Another complex cardiac disease associated with cardiac hypertrophy is "hypertrophic cardiomyopathy". This condition is characterized by a great diversity of morphologic, functional, and clinical features (Maron *et al.*, N. Engl. J. Med., 316: 780-789 (1987); Spirito *et al.*, N. Engl. J. Med., 320: 749-755 (1989); Louie and Edwards, Prog. Cardiovasc. Dis., 36: 275-308 (1994); Wigle *et al.*, Circulation, 92: 1680-1692 (1995)), the heterogeneity of which is accentuated by the fact that it afflicts patients of all ages. Spirito *et al.*, N. Engl. J. Med., 336: 775-785 (1997). The causative factors of hypertrophic cardiomyopathy are also diverse and little understood. In general, mutations in genes encoding sarcomeric proteins are associated with hypertrophic cardiomyopathy. Recent data suggest that β-myosin heavy chain mutations may account for approximately 30 to 40 percent of cases of familial hypertrophic cardiomyopathy. Watkins *et al.*, N. Engl. J. Med., 326: 1108-1114 (1992); Schwartz *et al*, Circulation, 91: 532-540 (1995); Marian and Roberts, Circulation, 92: 1336-1347 (1995); Thierfelder *et al.*, Cell, 77: 701-712 (1994); Watkins *et al.*, Nat. Gen., 11: 434-437 (1995). Besides β-myosin heavy chain, other locations of genetic mutations include cardiac troponin T, alpha topomyosin, cardiac myosin binding protein C, essential myosin light chain, and regulatory myosin light chain. See Malik and Watkins, Curr. Opin. Cardiol., 12: 295-302 (1997).

Supravalvular "aortic stenosis" is an inherited vascular disorder characterized by narrowing of the ascending aorta, but other arteries, including the pulmonary arteries, may also be affected. Untreated aortic stenosis may lead to increased intracardiac pressure resulting in myocardial hypertrophy and eventually heart failure and death. The pathogenesis of this disorder is not fully understood, but hypertrophy and possibly hyperplasia of medial smooth muscle are prominent features of this disorder. It has been reported that molecular variants of the elastin gene are involved in the development and pathogenesis of aortic stenosis. U.S. Patent No. 5,650,282 issued July 22, 1997.

"Valvular regurgitation" occurs as a result of heart diseases resulting in disorders of the cardiac valves. Various diseases,

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents, folic acid antagonists, anti-metabolites of nucleic acid metabolism, antibiotics, pyrimidine analogs, 5-fluorouracil, cisplatin, purine nucleosides, amines, amino acids, triazol nucleosides, or corticosteroids. Specific examples include Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Busulfan, Cytoxin, Taxol, Toxotere, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincreistine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins (see U.S. Pat. No. 4,675,187), Melphalan, and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors, such as tamoxifen and onapristone.

A "growth-inhibitory agent" when used herein refers to a compound or composition that inhibits growth of a cell, such as an Wnt-overexpressing cancer cell, either *in vitro* or *in vivo*. Thus, the growth-inhibitory agent is one which significantly reduces the percentage of malignant cells in S phase. Examples of growth-inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxol, and topo II inhibitors such as doxorubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in The Molecular Basis of Cancer, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami *et al.* (WB Saunders: Philadelphia, 1995), especially p. 13. Additional examples include tumor necrosis factor (TNF), an antibody capable of inhibiting or neutralizing the angiogenic activity of acidic or basic FGF or hepatocyte growth factor (HGF), an antibody capable of inhibiting or neutralizing the coagulant activities of tissue factor, protein C, or protein S (see

BOSENTAN™ and MOXONODIN™; interferon-gamma (IFN-γ); des-aspartate-angiotensin I; thrombolytic agents, *e.g.*, streptokinase, urokinase, t-PA, and a t-PA variant specifically designed to have longer half-life and very high fibrin specificity, TNK t-PA (a T103N, N117Q, KHRR(296-299)AAAA t-PA variant, Keyt *et al.*, Proc. Natl. Acad. Sci. USA 91, 3670-3674 (1994)); inotropic or hypertensive agents such as digoxigenin and β-adrenergic receptor blocking agents, *e.g.*, propranolol, timolol, tertalolol, carteolol, nadolol, betaxolol, penbutolol, acetobutolol, atenolol, metoprolol, and carvedilol; angiotensin converting enzyme (ACE) inhibitors, *e.g.*, quinapril, captopril, enalapril, ramipril, benazepril, fosinopril, and lisinopril; diuretics, *e.g.*, chorothiazide, hydrochlorothiazide, hydroflumethazide, methylchlothiazide, benzthiazide, dichlorphenamide, acetazolamide, and indapamide; and calcium channel blockers, *e.g.*, diltiazem, nifedipine, verapamil, nicardipine. One preferred category of this type is a therapeutic agent used for the treatment of cardiac hypertrophy or of a physiological condition instrumental in the development of cardiac hypertrophy, such as elevated blood pressure, aortic stenosis, or myocardial infarction.

"Angiogenic agents" and "endothelial agents" are active agents that promote angiogenesis and endothelial cell growth, respectively, or, if applicable, vasculogenesis. This would include factors that accelerate wound healing, such as growth hormone, insulin-like growth factor-I (IGF-I), VEGF, VIGF, PDGF, epidermal growth factor (EGF), CTGF and members of its family, FGF, and TGF-α and TGF-β.

"Angiostatic agents" are active agents that inhibit angiogenesis or vasculogenesis or otherwise inhibit or prevent growth of cancer cells. Examples include antibodies or other antagonists to angiogenic agents as defined above, such as antibodies to VEGF. They additionally include cytotherapeutic agents such as cytotoxic agents, chemotherapeutic agents, growth-inhibitory agents, apoptotic agents, and other agents to treat cancer, such as anti-HER-2, anti-CD20, and other bioactive and organic chemical agents.

In a pharmacological sense, in the context of the present invention, a "therapeutically effective amount" of an active agent (VEGF-E polypeptide or antagonist thereto) refers to an amount

The term "VEGF-E polypeptide receptor" as used herein refers to a cellular receptor for VEGF-E polypeptide, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as variants thereof that retain the ability to bind VEGF-E polypeptide.

The term "antibody" is used in the broadest sense and specifically covers single anti-VEGF-E polypeptide monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) and anti-VEGF-E antibody compositions with polyepitopic specificity. The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts.

"Active" or "activity" for the purposes herein refers to form(s) of VEGF-E which retain the biologic activities of native or naturally-occurring VEGF-E polypeptide.

Hybridization is preferably performed under "stringent conditions" which means (1) employing low ionic strength and high temperature for washing, for example, 0.015 sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C, or (2) employing during hybridization a denaturing agent, such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 nM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6/8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS. Yet another example is hybridization using a buffer of 10% dextran sulfate, 2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C. Other conditions previously described and well known can be used to arrive at high, low or moderate stringencies. When a nucleic acid sequence of a nucleic acid molecule is provided, other nucleic acid molecules hybridizing thereto under the conditions described above are considered within the scope of the sequence. Preferably, the nucleic acid sequence of a nucleic acid molecule as

plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. The plaques are hybridized with kinased synthetic primer at a temperature that permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques that hybridize with the probe are then selected and cultured, and the nucleic acid is recovered.

"Operably linked" refers to juxtaposition such that the normal function of the components can be performed. Thus, a coding sequence "operably linked" to control sequences refers to a configuration wherein the coding sequence can be expressed under the control of these sequences and wherein the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. For example, nucleic acid for a presequence or secretory leader is operably linked to nucleic acid for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

"Control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

"Expression system" refers to DNA sequences containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed with these sequences are capable of producing the encoded proteins. To effect transformation, the expression system may be included on a vector; however, the relevant DNA may then also be integrated into the host chromosome.

two restriction-cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5'-terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional (Maniatis *et al.*, Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory, 1982), pp. 133-134).

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see Lawn *et al.*, Nucleic Acids Res., 9: 6103-6114 (1981), and Goeddel *et al.*, Nucleic Acids Res., 8, 4057 (1980).

"Southern Analysis" is a method by which the presence of DNA sequences in a digest or DNA-containing composition is confirmed by hybridization to a known, labelled oligonucleotide or DNA fragment. For the purposes herein, unless otherwise provided, Southern analysis shall mean separation of digests on 1 percent agarose, denaturation, and transfer to nitrocellulose by the method of Southern, J. Mol. Biol., 98: 503-517 (1975), and hybridization as described by Maniatis *et al.*, Cell, 15: 687-701 (1978).

"Ligation" refers to the process of forming phosphodiester bonds between two double-stranded nucleic acid fragments (Maniatis *et al.*, 1982, *supra*, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 mg of approximately equimolar amounts of the DNA fragments to be ligated.

"Preparation" of DNA from transformants means isolating plasmid DNA from microbial culture. Unless otherwise provided, the alkaline/SDS method of Maniatis *et al.* 1982, *supra*, p. 90, may be used.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or

in U.S. Patent No. 5,364,934. Variations may be a substitution, deletion, or insertion of one or more codons encoding the VEGF-E polypeptide that results in a change in the amino acid sequence of the VEGF-E polypeptide as compared with the native-sequence VEGF-E. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the VEGF-E polypeptide. Guidance in determining which amino acid residue may be inserted, substituted, or deleted without adversely affecting the desired activity may be found by comparing the sequence of the VEGF-E polypeptide with that of homologous known protein molecules and minimizing the number of amino acid sequence changes made in regions of high homology. Amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with a serine, i.e., conservative amino acid replacements. Insertions or deletions may optionally be in the range of 1 to 5 amino acids. The variation allowed may be determined by systematically making insertions, deletions, or substitutions of amino acids in the sequence and testing the resulting variants for activity in the *in vitro* assays described in the Examples below.

The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis (Carter *et al.*, Nucl. Acids Res., 13:4331 (1986); Zoller *et al.*, Nucl. Acids Res., 10:6487 (1987)), cassette mutagenesis (Wells *et al.*, Gene, 34:315 (1985)), restriction selection mutagenesis (Wells *et al.*, Philos. Trans. R. Soc. London SerA, 317:415 (1986)), or other known techniques can be performed on the cloned DNA to produce the VEGF-E-encoding variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also typically preferred because it is

glycosylation sites that are not present in the native-sequence VEGF-E polypeptide.

Addition of glycosylation sites to VEGF-E polypeptides may be accomplished by altering the amino acid sequence thereof. The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the native-sequence VEGF-E polypeptide (for O-linked glycosylation sites). The VEGF-E amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the VEGF-E polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids.

Another means of increasing the number of carbohydrate moieties on the VEGF-E polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, e.g., in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the VEGF-E polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin *et al.*, Arch. Biochem. Biophys., 259:52 (1987) and by Edge *et al.*, Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al.*, Meth. Enzymol., 138:350 (1987).

Another type of covalent modification of VEGF-E comprises linking the VEGF-E polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

VEGF-E polypeptides of the present invention may also be modified in a way to form chimeric molecules comprising a VEGF-E polypeptide fused to another, heterologous polypeptide or amino acid sequence. In one embodiment, such a chimeric molecule comprises a

phase techniques (see, e.g., Stewart *et al.*, Solid-Phase Peptide Synthesis, W.H. Freeman Co., San Francisco, CA (1969); Merrifield, J. Am. Chem. Soc., 85:2149-2154 (1963)). *In vitro* protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be accomplished, for instance, using an Applied Biosystems Peptide Synthesizer (Foster City, CA) using manufacturer's instructions. Various portions of VEGF-E polypeptides may be chemically synthesized separately and combined using chemical or enzymatic methods to produce a full-length VEGF-E polypeptide.

1. Isolation of DNA Encoding VEGF-E

DNA encoding a VEGF-E polypeptide may be obtained from a cDNA library prepared from tissue believed to possess the VEGF-E mRNA and to express it at a detectable level. Accordingly, human VEGF-E-encoding DNA can be conveniently obtained from a cDNA library prepared from human tissue, such as described in the Examples. The VEGF-E-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to a VEGF-E polypeptide or oligonucleotides of at least about 17-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding VEGF-E is to use PCR methodology (Sambrook *et al.*, *supra*; Dieffenbach *et al.*, PCR Primer:A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)).

The Examples below describe techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like ^{32}P-labeled ATP, biotinylation, or enzyme labeling. Hybridization conditions,

method of Graham and van der Eb, Virology, 52:456-457 (1978) can be employed. General aspects of mammalian cell host system transformations have been described in U.S. Patent No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, J. Bact., 130:946 (1977) and Hsiao *et al.*, Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, *e.g.*, polybrene or polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown *et al.*, Methods in Enzymology, 185:527-537 (1990) and Mansour *et al.*, Nature, 336:348-352 (1988).

Suitable host cells for cloning or expressing the DNA in the vectors herein include prokaryote, yeast, or higher eukaryote cells Suitable prokaryotes include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *E. coli*. Various *E. coli* strains are publicly available, such as *E. coli* K12 strain MM294 (ATCC 31,446); *E. coli* X1776 (ATCC 31,537); *E. coli* strain W3110 (ATCC 27,325) and K5 772 (ATCC 53,635).

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for VEGF-E-encoding vectors. *Saccharomyces cerevisiae* is a commonly used lower eukaryotic host microorganism.

Suitable host cells for the expression of glycosylated VEGF-E are derived from multicellular organisms. Examples of invertebrate cells include insect cells such as Drosophila S2 and Spodoptera Sf9, as well as plant cells. Examples of useful mammalian host cell lines include Chinese hamster ovary (CHO) and COS cells. More specific examples include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen Virol., 36:59 (1977)); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); and mouse mammary tumor (MMT 060562, ATCC CCL51). The

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV, or BPV) are useful for cloning vectors in mammalian cells.

Expression and cloning vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the VEGF-E-encoding nucleic acid, such as DHFR or thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub *et al.*, Proc. Natl. Acad. Sci. USA, 77:4216 (1980). A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, Nature, 282:39 (1979); Kingsman *et al.*, Gene, 7:141 (1979); Tschemper *et al.*, Gene, 10:157 (1980)). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, Genetics, 85:12 (1977)).

Expression and cloning vectors usually contain a promoter operably linked to the VEGF-E-encoding nucleic acid sequence to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems (Chang *et al.*, Nature, 275:615 (1978); Goeddel *et al.*, Nature, 281:544 (1979)), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776), and hybrid promoters such as the tac promoter (deBoer *et al.*, Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)). Promoters for

origin, and adenovirus enhancers. The enhancer may be spliced into the vector at a position 5' or 3' to the VEGF-E coding sequence, but is preferably located at a site 5' from the promoter.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding VEGF-E.

Still other methods, vectors, and host cells suitable for adaptation to the synthesis of VEGF-E polypeptides in recombinant vertebrate cell culture are described in Gething *et al.*, Nature, 293:620-625 (1981); Mantei *et al.*, Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

4. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native-sequence VEGF-E polypeptide or against a synthetic peptide

E. Uses for VEGF-E and Formulations

1. Assays for Cardiovascular, Endothelial, and Angiogenic Activity

Various assays can be used to test the polypeptide herein for cardiovascular, endothelial, and angiogenic activity. Such assays include those provided in the Examples below.

Assays for testing for endothelin antagonist activity, as disclosed in U.S. Pat. No. 5,773,414, include a rat heart ventricle binding assay where the polypeptide is tested for its ability to inhibit iodinized endothelin-1 binding in a receptor assay, an endothelin receptor binding assay testing for intact cell binding of radiolabeled endothelin-1 using rabbit renal artery vascular smooth muscle cells, an inositol phosphate accumulation assay where functional activity is determined in Rat-1 cells by measuring intracellular levels of second messengers, an arachidonic acid release assay that measures the ability of added compounds to reduce endothelin-stimulated arachidonic acid release in cultured vascular smooth muscles, *in vitro* (isolated vessel) studies using endothelium from male New Zealand rabbits, and *in vivo* studies using male Sprague-Dawley rats. Assays for tissue generation activity include, without limitation, those described in WO 95/16035 (bone, cartilage, tendon); WO 95/05846 (nerve, neuronal), and WO 91/07491 (skin, endothelium).

Assays for wound-healing activity include, for example, those described in Winter, Epidermal Wound Healing, Maibach, HI and Rovee, DT, eds. (Year Book Medical Publishers, Inc., Chicago), pp. 71-112, as modified by the article of Eaglstein and Mertz, J. Invest. Dermatol., 71: 382-384 (1978).

An assay to screen for a test molecule relating to a VEGF-E polypeptide that binds an endothelin B_1 (ETB_1) receptor polypeptide and modulates signal transduction activity involves providing a host cell transformed with a DNA encoding endothelin B_1 receptor polypeptide, exposing the cells to the test candidate, and measuring endothelin B_1 receptor signal transduction activity, as described, *e.g.*, in U.S. Pat. No. 5,773,223.

There are several cardiac hypertrophy assays. *In vitro* assays include induction of spreading of adult rat cardiac myocytes. In this assay, ventricular myocytes are isolated from a single (male

the needle. This approach is described, for example, in Rossi *et al.*, Am. Heart J., 124: 700-709 (1992) and O'Rourke and Reibel, P.S.E.M.B., 200: 95-100 (1992).

In yet another *in vivo* assay, the effect on cardiac hypertrophy following experimentally induced myocardial infarction (MI) is measured. Acute MI is induced in rats by left coronary artery ligation and confirmed by electrocardiographic examination. A sham-operated group of animals is also prepared as control animals. Earlier data have shown that cardiac hypertrophy is present in the group of animals with MI, as evidenced by an 18% increase in heart weight-to-body weight ratio. Lai *et al., supra*. Treatment of these animals with candidate blockers of cardiac hypertrophy, *e.g.*, VEGF-E polypeptide, provides valuable information about the therapeutic potential of the candidates tested. One further such assay test for induction of cardiac hypertrophy is disclosed in U.S. Pat. No. 5,773,415, using Sprague-Dawley rats.

For cancer, a variety of well-known animal models can be used to further understand the role of the genes identified herein in the development and pathogenesis of tumors, and to test the efficacy of candidate therapeutic agents, including antibodies and other antagonists of the native VEGF-E polypeptides, such as small-molecule antagonists. The *in vivo* nature of such models makes them particularly predictive of responses in human patients. Animal models of tumors and cancers (*e.g.*, breast cancer, colon cancer, prostate cancer, lung cancer, etc.) include both non-recombinant and recombinant (transgenic) animals. Non-recombinant animal models include, for example, rodent, *e.g.*, murine models. Such models can be generated by introducing tumor cells into syngeneic mice using standard techniques, *e.g.*, subcutaneous injection, tail vein injection, spleen implantation, intraperitoneal implantation, implantation under the renal capsule, or orthopin implantation, *e.g.*, colon cancer cells implanted in colonic tissue. See, *e.g.*, PCT publication No. WO 97/33551, published September 18, 1997.

Probably the most often used animal species in oncological studies are immunodeficient mice and, in particular, nude mice. The observation that the nude mouse with thymic hypo/aplasia could successfully act as a host for human tumor xenografts has lead to its widespread use for this purpose. The autosomal recessive *nu*

model of human colon cancer in nude mice has been described, for example, by Wang *et al.*, Cancer Research, 54: 4726-4728 (1994) and Too *et al.*, Cancer Research, 55: 681-684 (1995). This model is based on the so-called "METAMOUSE"™ sold by AntiCancer, Inc. (San Diego, California).

Tumors that arise in animals can be removed and cultured *in vitro*. Cells from the *in vitro* cultures can then be passaged to animals. Such tumors can serve as targets for further testing or drug screening. Alternatively, the tumors resulting from the passage can be isolated and RNA from pre-passage cells and cells isolated after one or more rounds of passage analyzed for differential expression of genes of interest. Such passaging techniques can be performed with any known tumor or cancer cell lines.

For example, Meth A, CMS4, CMS5, CMS21, and WEHI-164 are chemically induced fibrosarcomas of BALB/c female mice (DeLeo *et al.*, J. Exp. Med., 146: 720 (1977)), which provide a highly controllable model system for studying the anti-tumor activities of various agents. Palladino *et al.*, J. Immunol., 138: 4023-4032 (1987). Briefly, tumor cells are propagated *in vitro* in cell culture. Prior to injection into the animals, the cell lines are washed and suspended in buffer, at a cell density of about $10x10^6$ to $10x10^7$ cells/ml. The animals are then infected subcutaneously with 10 to 100 µl of the cell suspension, allowing one to three weeks for a tumor to appear.

In addition, the Lewis lung (3LL) carcinoma of mice, which is one of the most thoroughly studied experimental tumors, can be used as an investigational tumor model. Efficacy in this tumor model has been correlated with beneficial effects in the treatment of human patients diagnosed with small-cell carcinoma of the lung (SCCL). This tumor can be introduced in normal mice upon injection of tumor fragments from an affected mouse or of cells maintained in culture. Zupi *et al.*, Br. J. Cancer, 41: suppl. 4, 30 (1980). Evidence indicates that tumors can be started from injection of even a single cell and that a very high proportion of infected tumor cells survive. For further information about this tumor model see Zacharski, Haemostasis, 16: 300-320 (1986).

techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding VEGF-E. Techniques known in the art to introduce a transgene into such animals include pronucleic microinjection (U.S. Patent No. 4,873,191); retrovirus-mediated gene transfer into germ lines (*e.g.*, Van der Putten *et al.*, Proc. Natl. Acad. Sci. USA, 82: 6148-615 (1985)); gene targeting in embryonic stem cells (Thompson *et al.*, Cell, 56: 313-321 (1989)); electroporation of embryos (Lo, Mol. Cell. Biol., 3: 1803-1814 (1983)); and sperm-mediated gene transfer. Lavitrano *et al.*, Cell, 57: 717-73 (1989). For a review, see, for example, U.S. Patent No. 4,736,866. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for VEGF-E transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding VEGF-E introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding VEGF-E. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with its overexpression. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition.

For the purpose of the present invention, transgenic animals include those that carry the transgene only in part of their cells ("mosaic animals"). The transgene can be integrated either as a single transgene, or in concatamers, *e.g.*, head-to-head or head-to-tail tandems. Selective introduction of a transgene into a particular cell type is also possible by following, for example, the technique of Lasko *et al.*, Proc. Natl. Acad. Sci. USA, 89: 6232-636 (1992). The expression of the transgene in transgenic animals can be monitored by standard techniques. For example, Southern blot analysis or PCR amplification can be used to verify the integration of the transgene. The level of mRNA expression can then be analyzed using techniques such as *in situ* hybridization, Northern blot

tumor that is the most common oral malignancy of cats, accounting for over 60% of the oral tumors reported in this species. It rarely metastasizes to distant sites, although this low incidence of metastasis may merely be a reflection of the short survival times for cats with this tumor. These tumors are usually not amenable to surgery, primarily because of the anatomy of the feline oral cavity. At present, there is no effective treatment for this tumor. Prior to entry into the study, each cat undergoes complete clinical examination and biopsy, and is scanned by computed tomography (CT). Cats diagnosed with sublingual oral squamous cell tumors are excluded from the study. The tongue can become paralyzed as a result of such tumor, and even if the treatment kills the tumor, the animals may not be able to feed themselves. Each cat is treated repeatedly, over a longer period of time. Photographs of the tumors will be taken daily during the treatment period, and at each subsequent recheck. After treatment, each cat undergoes another CT scan. CT scans and thoracic radiograms are evaluated every 8 weeks thereafter. The data are evaluated for differences in survival, response, and toxicity as compared to control groups. Positive response may require evidence of tumor regression, preferably with improvement of quality of life and/or increased life span.

In addition, other spontaneous animal tumors, such as fibrosarcoma, adenocarcinoma, lymphoma, chondroma, or leiomyosarcoma of dogs, cats, and baboons can also be tested. Of these, mammary adenocarcinoma in dogs and cats is a preferred model as its appearance and behavior are very similar to those in humans. However, the use of this model is limited by the rare occurrence of this type of tumor in animals.

Other *in vitro* and *in vivo* cardiovascular, endothelial, and angiogenic tests known in the art are also suitable herein.

2. Tissue Distribution

The results of the cardiovascular, endothelial, and angiogenic assays herein can be verified by further studies, such as by determining mRNA expression in various human tissues.

As noted before, gene amplification and/or gene expression in various tissues may be measured by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas,

becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies preferably are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte that remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody that is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. See, *e.g.*, US Pat No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

For immunohistochemistry, the tissue sample may be fresh or frozen or may be embedded in paraffin and fixed with a preservative such as formalin, for example.

4. Cell-Based Tumor Assays

Cell-based assays and animal models for cardiovascular, endothelial, and angiogenic disorders, such as tumors, can be used to verify the findings of a cardiovascular, endothelial, and angiogenic assay herein, and further to understand the relationship between the genes identified herein and the development and pathogenesis of undesirable cardiovascular, endothelial, and angiogenic cell growth. The role of gene products identified herein in the development and pathology of undesirable cardiovascular, endothelial, and angiogenic cell growth, *e.g.*, tumor cells, can be tested by using cells or cells lines that have been identified as being stimulated or inhibited by the VEGF-E polypeptide herein. Such cells include, for example, those set forth in the Examples below.

In a different approach, cells of a cell type known to be involved in a particular cardiovascular, endothelial, and angiogenic disorder are transfected with the cDNAs herein, and the ability of

membranes that are implanted into the patient (see, *e.g.*, U.S. Pat. Nos. 4,892,538 and 5,283,187).

There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, or transferred *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, transduction, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. Transduction involves the association of a replication-defective, recombinant viral (preferably retroviral) particle with ā cellular receptor, followed by introduction of the nucleic acids contained by the particle into the cell. A commonly used vector for *ex vivo* delivery of the gene is a retrovirus.

The currently preferred *in vivo* nucleic acid transfer techniques include transfection with viral or non-viral vectors (such as adenovirus, lentivirus, Herpes simplex I virus, or adeno-associated virus (AAV)) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are, for example, DOTMA, DOPE, and DC-Chol; see, *e.g.*, Tonkinson *et al.*, Cancer Investigation, 14(1): 54-65 (1996)). The most preferred vectors for use in gene therapy are viruses, most preferably adenoviruses, AAV, lentiviruses, or retroviruses. A viral vector such as a retroviral vector includes at least one transcriptional promoter/enhancer or locus-defining element(s), or other elements that control gene expression by other means such as alternate splicing, nuclear RNA export, or post-translational modification of messenger. In addition, a viral vector such as a retroviral vector includes a nucleic acid molecule that, when transcribed in the presence of a gene encoding VEGF-E polypeptide, is operably linked thereto and acts as a translation initiation sequence. Such vector constructs also include a packaging signal, long terminal repeats (LTRs) or portions thereof, and positive and negative strand primer binding sites appropriate to the virus used (if these are not already present in the viral vector). In addition, such vector typically includes a signal sequence for secretion of the VEGF-E polypeptide from a host cell in which it is placed. Preferably the signal

Individuals carrying mutations in the gene encoding human VEGF-E polypeptide may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy, and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki *et al.*, Nature, 324: 163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding the VEGF-E polypeptide can be used to identify and analyze VEGF-E polypeptide mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled RNA encoding VEGF-E polypeptide, or alternatively, radiolabeled antisense DNA sequences encoding VEGF-E polypeptide. Perfectly matched sequences can be distinguished from mismatched duplexes by RNAse A digestion or by differences in melting temperatures.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamidine gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures. See, *e.g.*, Myers *et al.*, Science, 230: 1242 (1985).

Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNAse and S1 protection or the chemical cleavage method, for example, Cotton *et al.*, Proc. Natl. Acad. Sci. USA, 85: 4397-4401 (1985).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNAse protection, chemical cleavage, direct DNA sequencing, or the use of restriction enzymes, *e.g.*, restriction fragment length polymorphisms (RFLP), and Southern blotting of genomic DNA.

labels, including radionucleotides such as ^{32}P or ^{35}S, or enzymatic labels such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems. Labeled probes having a sequence complementary to that of the VEGF-E gene of the present invention can be used to screen libraries of human cDNA, genomic DNA, or mRNA to determine which members of such libraries the probe hybridizes to. Hybridization techniques are described in further detail in the Examples below.

The probes may also be employed in PCR techniques to generate a pool of sequences for identification of closely related VEGF-E sequences.

9. Chromosome Mapping

Nucleotide sequences encoding a VEGF-E polypeptide can also be used to construct hybridization probes for mapping the gene which encodes that VEGF-E polypeptide and for the genetic analysis of individuals with genetic disorders. The nucleotide sequence provided herein may be mapped to a chromosome and specific regions of a chromosome using known techniques, such as *in situ* hybridization, linkage analysis against known chromosomal markers, and hybridization screening with libraries.

For chromosome identification, the sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease. Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis for the 3' untranslated region is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

10. Screening Assays for Drug Candidates

Screening assays can be designed to find lead compounds that mimic the biological activity of a native VEGF-E or a receptor for VEGF-E. Such screening assays will include assays amenable to high-throughput screening of chemical libraries, making them particularly suitable for identifying small molecule drug candidates. Small molecules contemplated include synthetic organic or inorganic compounds. The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays and cell based assays, which are well characterized in the art.

Hence, this invention encompasses methods of screening compounds to identify those that mimic the VEGF-E polypeptide (agonists) or prevent the effect of the VEGF-E polypeptide (antagonists). Screening assays for antagonist drug candidates are designed to identify compounds that bind or complex with the VEGF-E polypeptides encoded by the genes identified herein, or otherwise interfere with the interaction of the encoded polypeptides with other cellular proteins. Such screening assays will include assays amenable to high-throughput screening of chemical libraries, making them particularly suitable for identifying small molecule drug candidates.

The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays, and cell- based assays, which are well characterized in the art.

All assays for antagonists are common in that they call for contacting the drug candidate with a VEGF-E polypeptide encoded by a nucleic acid identified herein under conditions and for a time sufficient to allow these two components to interact.

In binding assays, the interaction is binding and the complex formed can be isolated or detected in the reaction mixture. In a particular embodiment, the VEGF-E polypeptide encoded by the gene identified herein or the drug candidate is immobilized on a solid phase, e.g., on a microtiter plate, by covalent or non-covalent

protein-protein interaction. Colonies containing interacting polypeptides are detected with a chromogenic substrate for β-galactosidase. A complete kit (MATCHMAKER™) for identifying protein-protein interactions between two specific proteins using the two-hybrid technique is commercially available from Clontech. This system can also be extended to map protein domains involved in specific protein interactions as well as to pinpoint amino acid residues that are crucial for these interactions.

Compounds that interfere with the interaction of a gene encoding a VEGF-E polypeptide identified herein and other intra- or extracellular components can be tested as follows: usually a reaction mixture is prepared containing the product of the gene and the intra- or extracellular component under conditions and for a time allowing for the interaction and binding of the two products. To test the ability of a candidate compound to inhibit binding, the reaction is run in the absence and in the presence of the test compound. In addition, a placebo may be added to a third reaction mixture, to serve as positive control. The binding (complex formation) between the test compound and the intra- or extracellular component present in the mixture is monitored as described hereinabove. The formation of a complex in the control reaction(s) but not in the reaction mixture containing the test compound indicates that the test compound interferes with the interaction of the test compound and its reaction partner.

If the VEGF-E polypeptide has the ability to stimulate the proliferation of endothelial cells in the presence of the co-mitogen ConA, then one example of a screening method takes advantage of this ability. Specifically, in the proliferation assay, human umbilical vein endothelial cells are obtained and cultured in 96-well flat-bottomed culture plates (Costar, Cambridge, MA) and supplemented with a reaction mixture appropriate for facilitating proliferation of the cells, the mixture containing Con-A (Calbiochem, La Jolla, CA). Con-A and the compound to be screened are added and after incubation at 37°C, cultures are pulsed with ^{3}H-thymidine and harvested onto glass fiber filters (phD; Cambridge Technology, Watertown, MA). Mean ^{3}(H)thymidine incorporation (cpm) of triplicate cultures is determined using a liquid scintillation counter (Beckman Instruments, Irvine, CA). Significant ^{3}

would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the gene encoding the putative receptor.

In another assay for antagonists, mammalian cells or a membrane preparation expressing the receptor would be incubated with labeled VEGF-E polypeptide in the presence of the candidate compound. The ability of the compound to enhance or block this interaction could then be measured. The compositions useful in the treatment of cardiovascular, endothelial, and angiogenic disorders include, without limitation, antibodies, small organic and inorganic molecules, peptides, phosphopeptides, antisense and ribozyme molecules, triple-helix molecules, etc., that inhibit the expression and/or activity of the target gene product.

More specific examples of potential antagonists include an oligonucleotide that binds to the VEGF-E polypeptide, (poly)peptide immunoglobulin fusions, and, in particular, antibodies including, without limitation, poly- and monoclonal antibodies and antibody fragments, single-chain antibodies, anti-idiotypic antibodies, and chimeric or humanized versions of such antibodies or fragments, as well as human antibodies and antibody fragments. Alternatively, a potential antagonist may be a closely related protein, for example, a mutated form of the VEGF-E polypeptide that recognizes the receptor but imparts no effect, thereby competitively inhibiting the action of the VEGF-E polypeptide.

Another potential VEGF-E polypeptide antagonist is an antisense RNA or DNA construct prepared using antisense technology, where, *e.g.*, an antisense RNA or DNA molecule acts to block directly the translation of mRNA by hybridizing to targeted mRNA and preventing protein translation. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes the mature VEGF-E polypeptides herein, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix - see Lee *et al.*, Nucl. Acids Res., 6: 3073 (1979); Cooney *et al.*, Science, 241: 456 (1988);

11. Types of Cardiovascular, Endothelial, and Angiogenic Disorders to be Treated

The VEGF-E polypeptides, or agonists or antagonists thereto, that have activity in the cardiovascular, angiogenic, and endothelial assays described herein, and/or whose gene product has been found to be localized to the cardiovascular system, are likely to have therapeutic uses in a variety of cardiovascular, endothelial, and angiogenic disorders, including systemic disorders that affect vessels, such as diabetes mellitus. The VEGF-E molecules herein have a number of therapeutic uses associated with survival, proliferation and/or differention of cells. Such uses include the treatment of umbilical vein endothelial cells, in view of the demonstrated ability of VEGF-E to increase survival of human umbilical vein endothelial cells. Treatment may be needed if the vein were subjected to traumata, or situations wherein artificial means are employed to enhance the survival of the umbilical vein, for example, where it is weak, diseased, based on an artificial matrix, or in an artificial environment. Other physiological conditions that could be improved based on the selective mitogenic character of VEGF-E are also included herein. Uses also include the treatment of fibroblasts and myocytes, in view of the demonstrated ability of VEGF-E to induce proliferation of fibroblasts and hypertrophy in myocytes. In particular, VEGF-E can be used in wound healing, tissue growth and muscle generation and regeneration.

Their therapeutic utility could include diseases of the arteries, capillaries, veins, and/or lymphatics. Examples of treatments hereunder include treating muscle wasting disease, treating osteoporosis, aiding in implant fixation to stimulate the growth of cells around the implant and therefore facilitate its attachment to its intended site, increasing IGF stability in tissues or in serum, if applicable, and increasing binding to the IGF receptor (since IGF has been shown *in vitro* to enhance human marrow erythroid and granulocytic progenitor cell growth).

The VEGF-E polypeptides or agonists or antagonists thereto may also be employed to stimulate erythropoiesis or granulopoiesis, to stimulate wound healing or tissue regeneration and associated

or macular degeneration and proliferative vitreoretinopathy, rheumatoid arthritis, Crohn's disease, atherosclerosis, ovarian hyperstimulation, psoriasis, endometriosis associated with neovascularization, restenosis subsequent to balloon angioplasty, scar tissue overproduction, for example, that seen in a keloid that forms after surgery, fibrosis after myocardial infarction, or fibrotic lesions associated with pulmonary fibrosis.

If, however, the molecule inhibits angiogenesis, it would be expected to be used directly for treatment of the above conditions.

On the other hand, if the molecule stimulates angiogenesis it would be used itself (or an agonist thereof) for indications where angiogenesis is desired such as peripheral vascular disease, hypertension, inflammatory vasculitides, Reynaud's disease and Reynaud's phenomenon, aneurysms, arterial restenosis, thrombophlebitis, lymphangitis, lymphedema, wound healing and tissue repair, ischemia reperfusion injury, angina, myocardial infarctions such as acute myocardial infarctions, chronic heart conditions, heart failure such as congestive heart failure, and osteoporosis.

Specific types of diseases are described below, where the VEGF-E polypeptide herein or antagonists thereof may serve as useful for vascular-related drug targeting or as therapeutic targets for the treatment or prevention of the disorders. Atherosclerosis is a disease characterized by accumulation of plaques of intimal thickening in arteries, due to accumulation of lipids, proliferation of smooth muscle cells, and formation of fibrous tissue within the arterial wall. The disease can affect large, medium, and small arteries in any organ. Changes in endothelial and vascular smooth muscle cell function are known to play an important role in modulating the accumulation and regression of these plaques.

Hypertension is characterized by raised vascular pressure in the systemic arterial, pulmonary arterial, or portal venous systems. Elevated pressure may result from or result in impaired endothelial function and/or vascular disease.

Inflammatory vasculitides include giant cell arteritis, Takayasu's arteritis, polyarteritis nodosa (including the microangiopathic form), Kawasaki's disease, microscopic polyangiitis, Wegener's granulomatosis, and a variety of infectious-

tumor angiogenesis include breast carcinomas, lung carcinomas, gastric carcinomas, esophageal carcinomas, colorectal carcinomas, liver carcinomas, ovarian carcinomas, thecomas, arrhenoblastomas, cervical carcinomas, endometrial carcinoma, endometrial hyperplasia, endometriosis, fibrosarcomas, choriocarcinoma, head and neck cancer, nasopharyngeal carcinoma, laryngeal carcinomas, hepatoblastoma, Kaposi's sarcoma, melanoma, skin carcinomas, hemangioma, cavernous hemangioma, hemangioblastoma, pancreas carcinomas, retinoblastoma, astrocytoma, glioblastoma, Schwannoma, oligodendroglioma, medulloblastoma, neuroblastomas, rhabdomyosarcoma, osteogenic sarcoma, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumor, renal cell carcinoma, prostate carcinoma, abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

Age-related macular degeneration (AMD) is a leading cause of severe visual loss in the elderly population. The exudative form of AMD is characterized by choroidal neovascularization and retinal pigment epithelial cell detachment. Because choroidal neovascularization is associated with a dramatic worsening in prognosis, the VEGF-E polypeptides or antagonist thereto is expected to be useful in reducing the severity of AMD.

Healing of trauma such as wound healing and tissue repair is also a targeted use for the VEGF-E polypeptides herein or their antagonists. Formation and regression of new blood vessels is essential for tissue healing and repair. This category includes bone, cartilage, tendon, ligament, and/or nerve tissue growth or regeneration, as well as wound healing and tissue repair and replacement, and in the treatment of burns, incisions, and ulcers. A VEGF-E polypeptide or antagonist thereof that induces cartilage and/or bone growth in circumstances where bone is not normally formed has application in the healing of bone fractures and cartilage damage or defects in humans and other animals. Such a preparation employing a VEGF-E polypeptide or antagonist thereof may have prophylactic use in closed as well as open fracture reduction and also in the improved fixation of artificial joints. *De novo* bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma-induced, or oncologic, resection-

circumstances where such tissue is not normally formed has application in the healing of tendon or ligament tears, deformities, and other tendon or ligament defects in humans and other animals. Such a preparation may have prophylactic use in preventing damage to tendon or ligament tissue, as well as use in the improved fixation of tendon or ligament to bone or other tissues, and in repairing defects to tendon or ligament tissue. *De novo* tendon/ligament-like tissue formation induced by a composition of the VEGF-E polypeptide herein or antagonist thereto contributes to the repair of congenital, trauma-induced, or other tendon or ligament defects of other origin, and is also useful in cosmetic plastic surgery for attachment or repair of tendons or ligaments. The compositions herein may provide an environment to attract tendon- or ligament-forming cells, stimulate growth of tendon- or ligament-forming cells, induce differentiation of progenitors of tendon- or ligament-forming cells, or induce growth of tendon/ligament cells or progenitors *ex vivo* for return *in vivo* to effect tissue repair. The compositions herein may also be useful in the treatment of tendinitis, carpal tunnel syndrome, and other tendon or ligament defects. The compositions may also include an appropriate matrix and/or sequestering agent as a carrier as is well known in the art.

The VEGF-E polypeptide or its antagonist may also be useful for proliferation of neural cells and for regeneration of nerve and brain tissue, *i.e.*, for the treatment of central and peripheral nervous system disease and neuropathies, as well as mechanical and traumatic disorders, that involve degeneration, death, or trauma to neural cells or nerve tissue. More specifically, a VEGF-E polypeptide or its antagonist may be used in the treatment of diseases of the peripheral nervous system, such as peripheral nerve injuries, peripheral neuropathy and localized neuropathies, and central nervous system diseases, such as Alzheimer's, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager syndrome. Further conditions that may be treated in accordance with the present invention include mechanical and traumatic disorders, such as spinal cord disorders, head trauma, and cerebrovascular diseases such as stroke. Peripheral neuropathies resulting from chemotherapy or other medical therapies may also be treatable using a VEGF-E polypeptide herein or antagonist thereto.

12. Administration Protocols, Schedules, Doses, and Formulations

The molecules herein and agonists and antagonists thereto are pharmaceutically useful as a prophylactic and therapeutic agent for various disorders and diseases as set forth above.

The VEGF-E of the present invention can be formulated according to known methods to prepare pharmaceutically-useful compositions, whereby the VEGF-E hereof is combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable carrier vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo *et al.* The VEGF-E herein may be administered parenterally to subjects suffering from cardiovascular diseases or conditions, or by other methods that ensure its delivery to the bloodstream in an effective form.

Compositions particularly well suited for the clinical administration of VEGF-E hereof employed in the practice of the present invention include, for example, sterile aqueous solutions, or sterile hydratable powders such as lyophilized protein. It is generally desirable to include further in the formulation an appropriate amount of a pharmaceutically acceptable salt, generally in an amount sufficient to render the formulation isotonic. A pH regulator such as arginine base, and phosphoric acid, are also typically included in sufficient quantities to maintain an appropriate pH, generally from 5.5 to 7.5. Moreover, for improvement of shelf-life or stability of aqueous formulations, it may also be desirable to include further agents such as glycerol. In this manner, variant VEGF-E formulations are rendered appropriate for parenteral administration, and, in particular, intravenous administration.

Therapeutic compositions of the VEGF-E polypeptides or agonists or antagonists are prepared for storage by mixing the desired molecule having the appropriate degree of purity with optional pharmaceutically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, 16th edition, Oslo, A. ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers

articles can be used in modulating endothelial cell growth and angiogenesis. In addition, tumor invasion and metastasis may be modulated with these articles.

The VEGF-E to be used for therapeutic administration must be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). The VEGF-E ordinarily will be stored in lyophilized form or as an aqueous solution if it is highly stable to thermal and oxidative denaturation. The pH of the VEGF-E preparations typically will be about from 6 to 8, although higher or lower pH values may also be appropriate in certain instances. It will be understood that use of certain of the foregoing excipients, carriers, or stabilizers will result in the formation of salts of the VEGF-E.

An isotonifier may be present to ensure isotonicity of a liquid composition of the VEGF-E polypeptide or antagonist thereto, and includes polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol, and mannitol. These sugar alcohols can be used alone or in combination. Alternatively, sodium chloride or other appropriate inorganic salts may be used to render the solutions isotonic.

The buffer may, for example, be an acetate, citrate, succinate, or phosphate buffer depending on the pH desired. The pH of one type of liquid formulation of this invention is buffered in the range of about 4 to 8, preferably about physiological pH.

The preservatives phenol, benzyl alcohol and benzethonium halides, *e.g.*, chloride, are known antimicrobial agents that may be employed.

Therapeutic VEGF-E polypeptide compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle. The formulations are preferably administered as repeated intravenous (i.v.), subcutaneous (s.c.), or intramuscular (i.m.) injections, or as aerosol formulations suitable for intranasal or intrapulmonary delivery (for intrapulmonary delivery see, *e.g.*, EP 257,956).

VEGF-E polypeptide can also be administered in the form of sustained-released preparations. Suitable examples of sustained-

The therapeutically effective dose of VEGF-E polypeptide or antagonist thereto will, of course, vary depending on such factors as the pathological condition to be treated (including prevention), the method of administration, the type of compound being used for treatment, any co-therapy involved, the patient's age, weight, general medical condition, medical history, etc., and its determination is well within the skill of a practicing physician. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the maximal therapeutic effect. If the VEGF-E polypeptide has a narrow host range, for the treatment of human patients formulations comprising human VEGF-E polypeptide, more preferably native-sequence human VEGF-E polypeptide, are preferred. The clinician will administer VEGF-E polypeptide until a dosage is reached that achieves the desired effect for treatment of the condition in question. For example, if the objective is the treatment of CHF, the amount would be one that inhibits the progressive cardiac hypertrophy associated with this condition. The progress of this therapy is easily monitored by echo cardiography. Similarly, in patients with hypertrophic cardiomyopathy, VEGF-E polypeptide can be administered on an empirical basis.

With the above guidelines, the effective dose generally is within the range of from about 0.001 to about 1.0 mg/kg, more preferably about 0.01-1 mg/kg, most preferably about 0.01-0.1 mg/kg.

For non-oral use in treating human adult hypertension, it is advantageous to administer VEGF-E polypeptide in the form of an injection at about 0.01 to 50 mg, preferably about 0.05 to 20 mg, most preferably 1 to 20 mg, per kg body weight, 1 to 3 times daily by intravenous injection. For oral administration, a molecule based on the VEGF-E polypeptide is preferably administered at about 5 mg to 1 g, preferably about 10 to 100 mg, per kg body weight, 1 to 3 times daily. It should be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less than 0.5 ng/mg protein. Moreover, for human administration, the formulations preferably meet sterility, pyrogenicity, general safety, and purity as required by FDA Office and Biologics standards.

administering the composition topically, systemically, or locally as an implant or device. When administered, the therapeutic composition for use is in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of bone, cartilage, or tissue damage. Topical administration may be suitable for wound healing and tissue repair. Preferably, for bone and/or cartilage formation, the composition would include a matrix capable of delivering the protein-containing composition to the site of bone and/or cartilage damage, providing a structure for the developing bone and cartilage and preferably capable of being resorbed into the body. Such matrices may be formed of materials presently in use for other implanted medical applications.

The choice of matrix material is based on biocompatibility, biodegradabiity, mechanical properties, cosmetic appearance, and interface properties. The particular application of the compositions will define the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined calcium sulfate, tricalcium phosphate, hydroxyapatite, polylactic acid, polyglycolic acid, and polyanhydrides. Other potential materials are biodegradable and biologically well-defined, such as bone or dermal collagen. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are nonbiodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics. Matrices may be comprised of combinations of any of the above-mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalcium phosphate. The bioceramics may be altered in composition, such as in calcium-aluminate-phosphate and processing to alter pore size, particle size, particle shape, and biodegradability.

One specific embodiment is a 50:50 (mole weight) copolymer of lactic acid and glycolic acid in the form of porous particles having diameters ranging from 150 to 800 microns. In some applications, it will be useful to utilize a sequestering agent, such as carboxymethyl cellulose or autologous blood clot, to prevent the polypeptide compositions from disassociating from the matrix.

hydroxypropyl methylcellulose, and hydroxypropyl cellulose; starch and fractionated starch; agar; alginic acid and alginates; gum arabic; pullullan; agarose; carrageenan; dextrans; dextrins; fructans; inulin; mannans; xylans; arabinans; chitosans; glycogens; glucans; and synthetic biopolymers; as well as gums such as xanthan gum; guar gum; locust bean gum; gum arabic; tragacanth gum; and karaya gum; and derivatives and mixtures thereof. The preferred gelling agent herein is one that is inert to biological systems, nontoxic, simple to prepare, and not too runny or viscous, and will not destabilize the VEGF-E held within it.

Preferably the polysaccharide is an etherified cellulose derivative, more preferably one that is well defined, purified, and listed in USP, e.g., methylcellulose and the hydroxyalkyl cellulose derivatives, such as hydroxypropyl cellulose, hydroxyethyl cellulose, and hydroxypropyl methylcellulose. Most preferred herein is methylcellulose.

The polyethylene glycol useful for gelling is typically a mixture of low- and high-molecular-weight polyethylene glycols to obtain the proper viscosity. For example, a mixture of a polyethylene glycol of molecular weight 400-600 with one of molecular weight 1500 would be effective for this purpose when mixed in the proper ratio to obtain a paste.

The term "water soluble" as applied to the polysaccharides and polyethylene glycols is meant to include colloidal solutions and dispersions. In general, the solubility of the cellulose derivatives is determined by the degree of substitution of ether groups, and the stabilizing derivatives useful herein should have a sufficient quantity of such ether groups per anhydroglucose unit in the cellulose chain to render the derivatives water soluble. A degree of ether substitution of at least 0.35 ether groups per anhydroglucose unit is generally sufficient. Additionally, the cellulose derivatives may be in the form of alkali metal salts, for example, the Li, Na, K, or Cs salts.

If methylcellulose is employed in the gel, preferably it comprises about 2-5%, more preferably about 3%, of the gel and the VEGF-E is present in an amount of about 300-1000 mg per ml of gel.

instructions for dosage, administration, adverse effects, contraindications, etc. See, *e.g.*, Physicians' Desk Reference (Medical Economics Data Production Co.: Montvale, N.J., 1997), 51th Edition.

Preferred candidates for combination therapy in the treatment of hypertrophic cardiomyopathy are β-adrenergic-blocking drugs (*e.g.*, propranolol, timolol, tertalolol, carteolol, nadolol, betaxolol, penbutolol, acetobutolol, atenolol, metoprolol, or carvedilol), verapamil, difedipine, or diltiazem. Treatment of hypertrophy associated with high blood pressure may require the use of antihypertensive drug therapy, using calcium channel blockers, *e.g.*, diltiazem, nifedipine, verapamil, or nicardipine; β-adrenergic blocking agents; diuretics, *e.g.*, chorothiazide, hydrochlorothiazide, hydroflumethazide, methylchlothiazide, benzthiazide, dichlorphenamide, acetazolamide, or indapamide; and/or ACE-inhibitors, *e.g.*, quinapril, captopril, enalapril, ramipril, benazepril, fosinopril, or lisinopril.

For other indications, VEGF-E polypeptides or their antagonists may be combined with other agents beneficial to the treatment of the bone and/or cartilage defect, wound, or tissue in question. These agents include various growth factors such as EGF, PDGF, TGF-α or TGF-β, IGF, FGF, and CTGF.

In addition, VEGF-E polypeptides or their antagonists used to treat cancer may be combined with cytotoxic, chemotherapeutic, or growth-inhibitory agents as identified above. Also, for cancer treatment, the VEGF-E polypeptide or antagonist thereof is suitably administered serially or in combination with radiological treatments, whether involving irradiation or administration of radioactive substances.

The effective amounts of the therapeutic agents administered in combination with VEGF-E polypeptide or antagonist thereof will be at the physician's or veterinarian's discretion. Dosage administration and adjustment is done to achieve maximal management of the conditions to be treated. For example, for treating hypertension, these amounts ideally take into account use of diuretics or digitalis, and conditions such as hyper- or hypotension, renal impairment, etc. The dose will additionally depend on such factors as the type of the therapeutic agent to be

1. Polyclonal Antibodies

The anti-VEGF-E antibodies of the present invention may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the VEGF-E polypeptide or a fusion protein thereof. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation.

2. Monoclonal Antibodies

The anti-VEGF-E antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, Nature, 256:495 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the VEGF-E polypeptide or a fusion protein thereof. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103). Immortalized cell lines are usually transformed

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567) or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy-chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof,

CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries (Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks *et al.*, J. Mol. Biol., 222:581 (1991)). The techniques of Colē *et al.* and Boerner *et al.* are also available for the preparation of human monoclonal antibodies (Cole *et al.*, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner *et al.*, J. Immunol., 147(1):86-95 (1991)).

4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for a VEGF-E polypeptide, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, Nature, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker *et al.*, EMBO J., 10:3655-3659 (1991).

Antibody variable domains with the desired binding specificities (antibody-antigen combining sites) can be fused to

enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al.* Cancer Research, 53: 2560-2565 (1993). Alternatively, an antibody can be engineered that has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al.*, Anti-Cancer Drug Design 3: 219-230 (1989).

7. Immunoconjugates

The invention also pertains to immunoconjugates comprising an antibody conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.*, an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (*i.e.*, a radioconjugate). Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof that can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y, and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al.*, Science, 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary

homogeneous phases (Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158). The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter *et al.*, Nature, 144:945 (1962); David *et al.*, Biochemistry, 13:1014 (1974); Pain *et al.*, J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

Anti-VEGF-E antibodies also are useful for the affinity purification of VEGF-E polypeptides from recombinant cell culture or natural sources. In this process, the antibodies against a VEGF-E polypeptide are immobilized on a suitable support, such as Sephadex™ resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the VEGF-E polypeptide to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the VEGF-E polypeptide, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the VEGF-E polypeptide from the antibody.

1. Pharmaceutical Compositions of Antibodies

Antibodies specifically binding a VEGF-E polypeptide identified herein, as well as other molecules identified by the screening assays disclosed hereinbefore, can be administered for the treatment of various disorders as noted above and below in the form of pharmaceutical compositions.

If the VEGF-E polypeptide is intracellular and whole antibodies are used as inhibitors, internalizing antibodies are preferred. However, lipofections or liposomes can also be used to deliver the antibody, or an antibody fragment, into cells. Where

leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

2. Methods of Treatment using the Antibody

It is contemplated that the antibodies to VEGF-E polypeptide may be used to treat various cardiovascular, endothelial, and angiogenic conditions as noted above.

The antibodies are administered to a mammal, preferably a human, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous administration of the antibody is preferred.

Other therapeutic regimens may be combined with the administration of the antibodies of the instant invention as noted above. For example, if the antibodies are to treat cancer, the patient to be treated with such antibodies may also receive radiation therapy. Alternatively, or in addition, a chemotherapeutic agent may be administered to the patient. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service, Ed., M.C. Perry (Williams & Wilkins: Baltimore, MD, 1992). The chemotherapeutic agent may precede, or

anti-protein C antibody, anti-protein S antibody, or C4b binding protein (see WO 91/01753, published 21 February 1991), or heat or radiation.

Since the auxiliary agents will vary in their effectiveness, it is desirable to compare their impact on the tumor by matrix screening in conventional fashion. The administration of anti-VEGF-E polypeptide antibody and TNF is repeated until the desired clinical effect is achieved. Alternatively, the anti-VEGF-E polypeptide antibody is administered together with TNF and, optionally, auxiliary agent(s). In instances where solid tumors are found in the limbs or in other locations susceptible to isolation from the general circulation, the therapeutic agents described herein are administered to the isolated tumor or organ. In other embodiments, a FGF or PDGF antagonist, such as an anti-FGF or an anti-PDGF neutralizing antibody, is administered to the patient in conjunction with the anti-VEGF-E polypeptide antibody. Treatment with anti-VEGF-E polypeptide antibodies preferably may be suspended during periods of wound healing or desirable neovascularization.

For the prevention or treatment of cardiovascular, endothelial, and angiogenic disorder, the appropriate dosage of an antibody herein will depend on the type of disorder to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

For example, depending on the type and severity of the disorder, about 1 µg/kg to 50 mg/kg (*e.g.*, 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily or weekly dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated or sustained until a desired suppression of disorder symptoms occurs. However, other dosage regimens may be useful. The progress of this

EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, Virginia.

EXAMPLE I: Identification of clones encoding a VEGF-related protein (VEGF-E)

Probes based on an expressed sequence tag (EST) identified from the Incyte Pharmaceuticals database due to homology with VEGF were used to screen a cDNA library derived from the human glioma cell line G61. In particular, Incyte Clone "INC1302516" was used to generate the following four probes:

(SEQ ID NO:3) 5'-ACTTCTCAGTGTCCATAAGGG;

(SEQ ID NO:4) 5'-GAACTAAAGAGAACCGATACCATTTTCTGGCCAGGTTGTC;

(SEQ ID NO:5) 5'-CACCACAGCGTTTAACCAGG; and

(SEQ ID NO:6) 5'-ACAACAGGCACAGTTCCCAC.

Nine positives were identified and characterized. Three clones contained the full coding region and were identical in sequence. Partial clones were also identified from a fetal lung library and were identical with the glioma-derived sequence with the exception of one nucleotide change, which did not alter the encoded amino acid.

EXAMPLE 2: Expression constructs

For mammalian protein expression, the entire open reading frame (ORF) was cloned into a CMV-based expression vector. An epitope-tag (FLAG™, Kodak) and Histidine-tag (His8) were inserted between the ORF and stop codon. VEGF-E-His8 and VEGF-E-FLAG were transfected into human embryonic kidney 293 cells by SuperFect™ (Qiagen) and pulse-labeled for 3 hours with (^{35}S)methionine and (^{35}C)cysteine. Both epitope-tagged proteins co-migrate when 20 microliters of 15-fold concentrated serum-free conditioned medium were electrophoresed on a polyacrylamide gel (Novex) in sodium dodecyl sulfate sample buffer (SDS-PAGE). The VEGF-E-IgG expression

In situ hybridization was performed following an optimized version of the protocol by Lu and Gillett, Cell Vision 1: 169-176 (1994), using PCR-generated ^{33}P-labeled riboprobes. Briefly, formalin-fixed, paraffin-embedded human tissues were sectioned, deparaffinized, deproteinated in proteinase K (20 g/ml) for 15 minutes at 37°C, and further processed for *in situ* hybridization as described by Lu and Gillett, *supra*. A (^{33}-P)UTP-labeled antisense riboprobe was generated from a PCR product of 980 bp (using the oligonucleotide primers indicated below) and hybridized at 55°C overnight. The slides were dipped in KODAK NTB2™ nuclear track emulsion and exposed for 4 weeks.

^{33}P-Riboprobe synthesis

6.0 µl (125 mCi) of ^{33}P-UTP (Amersham BF 1002, SA<2000 Ci/mmol) were speed-vacuum dried. To each tube containing dried ^{33}P-UTP, the following ingredients were added:

2.0 µl 5x transcription buffer

1.0 µl DTT (100 mM)

2.0 µl NTP mix (2.5 mM : 10 µl each of 10 mM GTP, CTP & ATP + 10 µl H_2O)

1.0 µl UTP (50 µM)

1.0 µl RNAsin

1.0 µl DNA template (1 µg)

1.0 µl H_2O

1.0 µl RNA polymerase (for PCR products T3 = AS, T7 = S, usually)

The tubes were incubated at 37°C for one hour. A total of 1.0 µl RQ1 DNase was added, followed by incubation at 37°C for 15 minutes. A total of 90 µl TE (10 mM Tris pH 7.6/1 mM EDTA pH 8.0) was added, and the mixture was pipetted onto DE81 paper. The remaining solution was loaded in a MICROCON-50™ ultrafiltration unit, and spun using program 10 (6 minutes). The filtration unit was inverted over a second tube and spun using program 2 (3 minutes). After the final recovery spin, a total of 100 µl TE was added. Then 1 µl of the final product was pipetted on DE81 paper and counted in 6 ml of BIOFLUOR II™.

The probe was run on a TBE/urea gel. A total of 1-3 µl of the probe or 5 µl of RNA Mrk III was added to 3 µl of loading buffer.

added, and the tissue was vortexed well and incubated at 42°C for 1-4 hours.

D. Hybridization

1.0 x 10^5 cpm probe and 1.0 µl tRNA (50 mg/ml stock) per slide were heated at 95°C for 3 minutes. The slides were cooled on ice, and 48 µl hybridization buffer was added per slide. After vortexing, 50 µl ^{33}P mix was added to 50 µl prehybridization on the slide. The slides were incubated overnight at 55°C.

E. Washes

Washing was done for 2x10 minutes with 2xSSC, EDTA at room temperature (400 ml 20 x SSC + 16 ml 0.25 M EDTA, V_f=4L), followed by RNAseA treatment at 37°C for 30 minutes (500 µl of 10 mg/ml in 250 ml RNAse buffer = 20 µg/ml). The slides were washed 2x10 minutes with 2 x SSC, EDTA at room temperature. The stringency wash conditions were as follows: 2 hours at 55°C, 0.1 x SSC, EDTA (20 ml 20 x SSC + 16 ml EDTA, V_f=4L).

F. Oligonucleotide Primers

In situ analysis was performed on the DNA29101 sequence disclosed herein. The oligonucleotide primers employed to prepare the riboprobe for these analyses were as follows.

p1: 5'-GGA TTC TAA TAC GAC TCA CTA TAG GGC GGC GGA ATC CAA CCT GAG TAG (SEQ ID NO:7)

p2 5'- CTA TGA AAT TAA CCC TCA CTA AAG GGA GCG GCT ATC CTC CTG TGC TC (SEQ ID NO:8)

G. Results

The results from this *in situ* analysis were as follows.

For the lower human fetal limb, there was expression of VEGF-E in developing lower limb bones at the edge of the cartilagenous anlage (i.e., around the outside edge), in developing tendons, in vascular smooth muscle, and in cells embracing developing skeletal muscle myocytes and myotubes. Expression was also observed at the epiphyseal growth plate. There was human fetal lymph node

placenta, umbilical cord, liver, kidney, adrenals, thyroid, lungs, heart, great vessels, oesophagus, stomach, small intestine, spleen, thymus, pancreas, brain, eye, spinal cord, body wall, pelvis and lower limb.

The adult tissues examined in the above study included: liver, kidney, adrenal, myocardium, aorta, spleen, lymph node, pancreas, lung, skin, cerebral cortex (rm), hippocampus (rm), cerebellum (rm), penis, eye, bladder, stomach, gastric carcinoma, colon, colonic carcinoma, and chondrosarcoma, as well as tissues having acetominophen-induced liver injury, and hepatic cirrhosis.

In summary, the expression pattern suggests that VEGF-E may be involved in cell differentiation and/or proliferation. Expression patterns in developing skeletal muscle suggest that the protein may be involved in myoblast differentiation and/or proliferation.

EXAMPLE 5: Myocyte hypertrophy assay

Myocytes from neonatal Harlan Sprague Dawley rat heart ventricle (23 days gestation) were plated in duplicate at 75000 cells/ml in a 96-well plate. Cells were treated for 48h with 2000, 200, 20, or 2 ng/ml VEGF-E-IgG. Myocytes were stained with crystal violet to visualize morphology and scored on a scale of 3 to 7, 3 being nonstimulated and 7 being full-blown hypertrophy.

2000 ng/ ml and 200 ng/ ml VEGF-E caused hypertrophy, scored as a 5.

EXAMPLE 6: Cell proliferation assay

Mouse embryonic fibroblast C3H1OT1/2 cells (ATCC) were grown in 50:50 Ham's F-12: low glucose DMEM medium containing 10% fetal calf serum (FCS). Cells were plated in duplicate in a 24-well plate at 1000, 2000, and 4000 cells/well. After 48 hours, cells were switched to medium containing 2% FCS and were incubated for 72 hours with 200, 800, or 2000 ng/ml VEGF-E or no growth factor added.

Approximately 1.5 fold greater number of cells were measured in the presence of 200 ng/ml VEGF-E as in its absence, at all three cell densities.

EXAMPLE 7: Endothelial cell survival assay

A positive result is equal to or greater than 2. 1 = vacuoles present in less than 20% of cells, 2 = vacuoles present in 20-50% of cells, 3 = vacuoles present in greater than 50% of cells. This assay is designed to identify factors that are involved in stimulating pinocytosis, ion pumping, permeability, and junction formation.

3. Tube Formation Assay

This assay is to identify factors that stimulate endothelial tube formation in a 3-dimensional matrix. This assay will identify factors that stimulate endothelial cells to differentiate into a tube-like structure in a 3-dimensional matrix in the presence of exogenous growth factors (VEGF, bFGF).

A positive result is equal to or greater than 2. 1 = cells are all round, 2 = cells are elongated, 3 = cells are forming tubes with some connections, 4 = cells are forming complex tubular networks. This assay would identify factors that may be involved in stimulating tracking, chemotaxis, or endothelial shape change.

The results are shown in Figures 3 through 5. Fig. 3A shows the HUVEC tube formation when no growth factors are present. Fig. 3B shows where VEGF/bFGF, and PMA are present, Fig. 3C shows where VEGF and bFGF are present, Fig. 3D shows where VEGF and PMA are present, Fig. 3E shows where bFGF and PMA are present, Fig. 3F shows where VEGF is present, Fig. 3G shows where bFGF is present, and Fig. 3H shows where PMA is present.

Figs. 4A and 4B show, respectively, the effect on HUVEC tube formation of VEGF-E-IgG at 1% dilution and of a buffer control (10 mM HEPES/0.14M NaCl/4% mannitol, pH 6.8) at 1% dilution. Figs. 5A and 5B show, respectively, the effect on HUVEC tube formation of VEGF-E-poly-his at 1% dilution and of the buffer control used for VEGF-E-IgG at 1% dilution.

The results clearly show more complex tube formation with the VEGF-E-IgG and VEGF-E-poly-his samples than with the buffer controls.

EXAMPLE 9: Transgenic mice

Transgenic mice were generated by microinjection of C57Bl/6/SJL F2 mouse embryos (DNAX) with a vector suitable for such microinjection containing the cDNA encoding VEGF-E under the control

the wells was aspirated, and the cells were washed 1X with PBS. An acid phosphatase reaction mixture (100 microliter; 0.1M sodium acetate, pH 5.5, 0.1% Triton X-100, 10 mM p-nitrophenyl phosphate) was then added to each well. After a 2 hour incubation at 37°C, the reaction was stopped by addition of 10 microliters 1N NaOH. Optical density (OD) was measured on a microplate reader at 405 nm.

The activity of VEGF-E was calculated as the percent inhibition of VEGF (3 ng/ml) stimulated proliferation (as determined by the acid phosphatase activity at OD 405 nm) relative to the cells without stimulation. TGF-beta was employed as an activity reference- at 1 ng/ml, TGF-beta blocks 70-90% of VEGF-stimulated ACE cell proliferation. Results of the assay were interpreted as "positive" if the observed inhibition was $\geq$30%.

In a first assay run, the VEGF-E at 1%, 0.1%, and 0.01% dilutions exhibited 52%, 90% and 96% inhibition, respectively. In a second assay run, the VEGF-E at 1%, 0.1%, and 0.01% dilutions exhibited 57%, 93% and 91% inhibition, respectively.

Deposit of material

The following material has been deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, Virginia USA (ATCC):

Material	ATCC Dep. No.	Deposit Date
DNA29101-1272	209653	March 5, 1998

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to

WHAT IS CLAIMED IS:

1. An isolated nucleic acid comprising a nucleotide sequence encoding a vascular endothelial cell growth factor-E (VEGF-E) polypeptide comprising amino acid residues 1 through 345 of Figure 2 (SEQ ID NO:2).

2. The nucleic acid of claim 1 comprising the sequence of nucleotides 259 through 1293 of Figure 1 (SEQ ID NO:1), or its complement.

3. An isolated nucleic acid comprising a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of claim 1.

4. A vector comprising the nucleic acid of claim 1.

5. A host cell comprising the nucleic acid of claim 1.

6. The host cell of claim 5, wherein said cell is a Chinese Hamster Ovary cell, an insect cell, an *E. coli* cell, or a yeast cell.

7. The host cell of claim 6 that is a baculovirus-infected insect cell.

8. A process for producing a vascular endothelial cell growth factor-E (VEGF-E) polypeptide comprising culturing the host cell of claim 5 under conditions suitable for expression of the VEGF-E polypeptide and recovering the VEGF-E polypeptide from the cell culture.

9. A polypeptide produced by the process of claim 8.

10. A VEGF-E polypeptide comprising amino acid residues 1 through 345 of Figure 2 (SEQ ID NO:2).

11. A VEGF-E polypeptide selected from the group consisting of:

21. The method of claim 19 wherein said cardiac hypertrophy has been induced by myocardial infarction.

22. The method of claim 21 wherein said VEGF-E polypeptide administration is initiated within 48 hours following myocardial infarction.

23. A method for diagnosing a disease or a susceptibility to a disease related to a mutation in a nucleic acid sequence encoding vascular endothelial cell growth factor-E (VEGF-E) comprising:

(a) isolating a nucleic acid sequence encoding VEGF-E from a sample derived from a host; and

(b) determining a mutation in the nucleic acid sequence encoding VEGF-E.

24. A method of diagnosing cardiovascular and endothelial disorders in a mammal comprising detecting the level of expression of a gene encoding a vascular endothelial cell growth factor-E (VEGF-E) polypeptide (a) in a test sample of tissue cells obtained from the mammal, and (b) in a control sample of known normal tissue cells of the same cell type, wherein a higher or lower expression level in the test sample indicates the presence of a cardiovascular or endothelial dysfunction in the mammal from which the test tissue cells were obtained.

25. A method for identifying an agonist to a vascular endothelial cell growth factor-E (VEGF-E) polypeptide comprising:

(a) contacting cells and a candidate compound under conditions that allow the polypeptide to stimulate proliferation of the cells; and

(b) measuring the extent to which cell proliferation is inhibited by the compound.

26. An agonist to a VEGF-E polypeptide identified by the method of claim 25.

27. A method for identifying a compound that inhibits the expression or activity of a vascular endothelial cell growth factor-E (VEGF-E) polypeptide, comprising:

(a) contacting a candidate compound with the polypeptide under conditions and for a time sufficient to allow the compound and polypeptide to interact; and

GACGCGTGGGCGGACGCGTGGGCTGGTTCAGGTCCAGGTTTTGCTTTGATCCTTTTCAAA
AACTGGAGACACAGAAGAGGGCTCTAGGAAAAAGTTTTGGATGGGATTATGTGGAAACTA
CCCTGCGATTCTCTGCTGCCAGAGCAGGCTCGGCGCTTCCACCCCAGTGCAGCCTTCCCC
TGGCGGTGGTGAAAGAGACTCGGGAGTCGCTGCTTCCAAAGTGCCCGCCGTGAGTGAGCT
CTCACCCCAGTCAGCCAA

ATGAGCCTCTTCGGGCTTCTCCTGCTGACATCTGCCCTGGCCGGCCAGAGACAGGGGACT
CAGGCGGAATCCAACCTGAGTAGTAAATTCCAGTTTTCCAGCAACAAGGAACAGAACGGA
GTACAAGATCCTCAGCATGAGAGAATTATTACTGTGTCTACTAATGGAAGTATTCACAGC
CCAAGGTTTCCTCATACTTATCCAAGAAATACGGTCTTGGTATGGAGATTAGTAGCAGTA
GAGGAAAATGTATGGATACAACTTACGTTTGATGAAAGATTTGGGCTTGAAGACCCAGAA
GATGACATATGCAAGTATGATTTTGTAGAAGTTGAGGAACCCAGTGATGGAACTATATTA
GGGCGCTGGTGTGGTTCTGGTACTGTACCAGGAAAACAGATTTCTAAAGGAAATCAAATT
AGGATAAGATTTGTATCTGATGAATATTTTCCTTCTGAACCAGGGTTCTGCATCCACTAC
AACATTGTCATGCCACAATTCACAGAAGCTGTGAGTCCTTCAGTGCTACCCCCTTCAGCT
TTGCCACTGGACCTGCTTAATAATGCTATAACTGCCTTTAGTACCTTGGAAGACCTTATT
CGATATCTTGAACCAGAGAGATGGCAGTTGGACTTAGAAGATCTATATAGGCCAACTTGG
CAACTTCTTGGCAAGGCTTTTGTTTTTGGAAGAAAATCCAGAGTGGTGGATCTGAACCTT
CTAACAGAGGAGGTAAGATTATACAGCTGCACACCTCGTAACTTCTCAGTGTCCATAAGG
GAAGAACTAAAGAGAACCGATACCATTTTCTGGCCAGGTTGTCTCCTGGTTAAACGCTGT
GGTGGGAACTGTGCCTGTTGTCTCCACAATTGCAATGAATGTCAATGTGTCCCAAGCAAA
GTTACTAAAAAATACCACGAGGTCCTTCAGTTGAGACCAAAGACCGGTGTCAGGGGATTG
CACAAATCACTCACCGACGTGGCCCTGGAGCACCATGAGGAGTGTGACTGTGTGTGCAGA
GGGAGCACAGGAGGATAGCCGCATCACCACCAGCAGCTCTTGCCCAGAGCTGTGCAGTGC
AGTGGCTGATTCTATTAGAGAACGTATGCGTTATCTCCATCCTTAATCTCAGTTGTTTGC
TTCAAGGACCTTTCATCTTCAGGATTTACAGTGCATTCTGAAAGAGGAGACATCAAACAG
AATTAGGAGTTGTGCAACAGCTCTTTTGAGAGGAGGCCTAAAGGACAGGAGAAAAGGTCT
TCAATCGTGGAAAGAAAATTAAATGTTGTATTAAATAGATCACCAGCTAGTTTCAGAGTT
ACCATGTACGTATTCCACTAGCTGGGTTCTGTATTTCAGTTCTTTCGATACGGCTTAGGG
TAATGTCAGTACAGGAAAAAAACTGTGCAAGTGAGCACCTGATTCCGTTGCCTTGCTTAA
CTCTAAAGCTCCATGTCCTGGGCCTAAAATCGTATAAAATCTGGATTTTTTTTTTTTTTT
TTGCTCATATTCACATATGTAAACCAGAACATTCTATGTACTACAAACCTGGTTTTTAAA
AAGGAACTATGTTGCTATGAATTAAACTTGTGTCATGCTGATAGGACAGACTGGATTTTT
CATATTTCTTATTAAAATTTCTGCCATTTAGAAGAAGAGAACTACATTCATGGTTTGGAA
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GTTTCATTGTGTACATTTTTATATTCTCCTTTTGACATTATAACTGTTGGCTTTTCTAAT
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AGACTTTTTGAAAATAATTAAATTATCATATCTTCCATTCCTGTTATTGGAGATGAAAAT
AAAAAGCAACTTATGAAAGTAGACATTCAGATCCAGCCATTACTAACCTATTCCTTTTTT
GGGGAAATCTGAGCCTAGCTCAGAAAAACATAAAGCACCTTGAAAAAGACTTGGCAGCTT
CCTGATAAAGCGTGCTGTGCTGTGCAGTAGGAACACATCCTATTTATTGTGATGTTGTGG
TTTTATTATCTTAAACTCTGTTCCATACACTTGTATAAATACATGGATATTTTTATGTAC
AGAAGTATGTCTCTTAACCAGTTCACTTATTGTACTCTGGCAATTTAAAAGAAAATCAGT
AAAATATTTTGCTTGTAAAATGCTTAATATNGTGCCTAGGTTATGTGGTGACTATTTGAA
TCAAAAATGTATTGAATCATCAAATAAAAGAATGTGGCTATTTTGGGGAGAAAATTAAAA
AAAAAAAAAAAAAAAAAGGTTTAGGGATAACAGGGTAATGCGGCCGC SEQ. ID NO:1

FIG. 1

no growth factor(s)

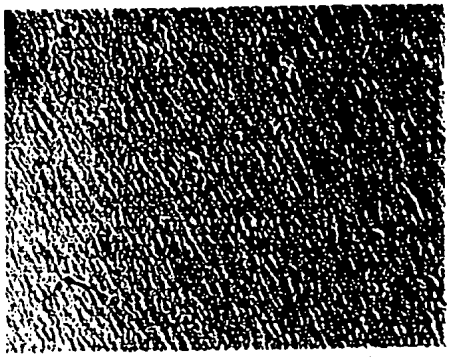

FIG. 3A

vegf/bfgf/pma

FIG. 3B

vegf/bfgf

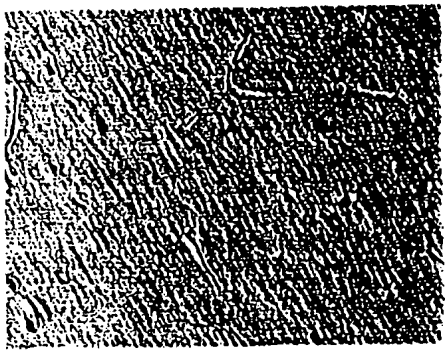

FIG. 3C

vegf/pma

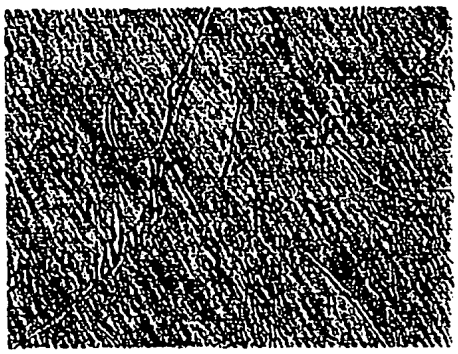

FIG. 3D

Buffer control @ 1% dilution

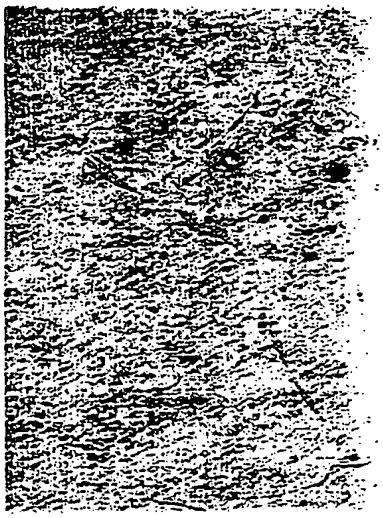

FIG. 4B

+VEGF-E-IgG @ 1% dilution

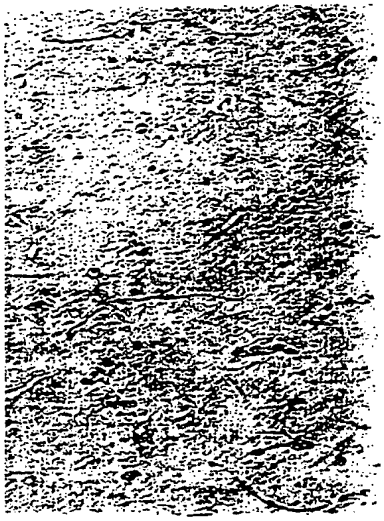

FIG. 4A

Buffer control @ 1% dilution

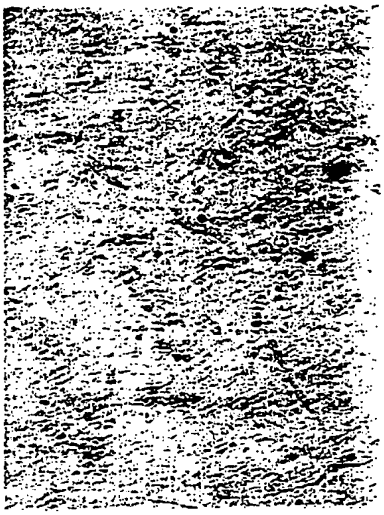

FIG. 5B

+VEGF-E-poly-His @ 1% dilution

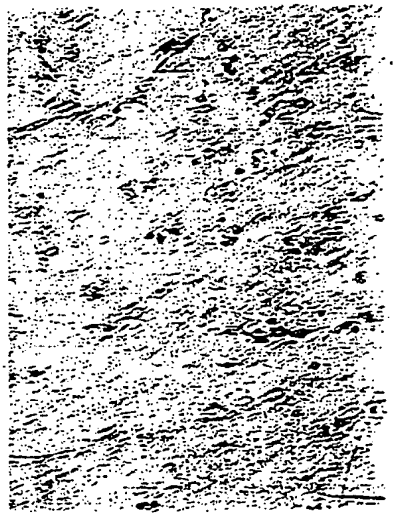

FIG. 5A

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International Bureau

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(54) Title: POLYPEPTIDES HOMOLOGOUS TO VEGF AND BMP1

(57) Abstract

The present invention involves the identification and preparation of vascular endothelial growth factor–E (VEGF–E). VEGF–E is a novel polypeptide related to vascular endothelial growth factor (VEGF) and bone morphogenetic protein 1. VEGF–E has homology to VEGF including conservation of the amino acids required for activity of VEGF. VEGF–E can be useful in wound repair, as well as in the generation and regeneration of tissue.

INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/US 99/05190

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/19 C12N15/62 C12N15/86 C12N5/10 C07K14/52
C07K16/24 C12Q1/68 A61K38/19 G01N33/50 G01N33/577
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K C12Q A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL Database, Heidelberg, FRG, Accession Number AA631149, 31/10/97 (Version 2) NCI-CGAP: "EST nq75h03.s1 NCI_CGAP_Pr22 Homo sapiens cDNA clone IMAGE:1158197" XP002113997 the whole document ---	3
A	ACHEN ET AL: "Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4)" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, vol. 95, January 1998 (1998-01), pages 548-553, XP002066364 abstract; figure 4 ---	1-25,27, 29-33
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 September 1999	16/09/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Jansen, K-S

3

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US 99/05190

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 18-22 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 26,28
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

See FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[illegible]tional Application No
PCT/US 99/05190

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9807832 A	26-02-1998	AU 4079697 A	06-03-1998
WO 8800205 A	14-01-1988	US 4877864 A	31-10-1989
		AT 141928 T	15-09-1996
		AU 613314 B	01-08-1991
		AU 7783587 A	29-01-1988
		DE 3751887 D	02-10-1996
		DE 3751887 T	06-03-1997
		DK 53497 A	09-05-1997
		DK 106288 A	28-04-1988
		EP 0313578 A	03-05-1989
		EP 0688869 A	27-12-1995
		GR 871028 A	11-01-1988
		IE 75881 B	24-09-1997
		IL 83003 A	31-07-1995
		JP 2729222 B	18-03-1998
		JP 6298800 A	25-10-1994
		JP 10070989 A	17-03-1998
		JP 2500241 T	01-02-1990
		JP 2713715 B	16-02-1998
		KR 9705583 B	18-04-1997
		MX 170919 B	22-09-1993
		PT 85225 A,B	01-08-1987
		US 5543394 A	06-08-1996
		US 5631142 A	20-05-1997
		US 5013649 A	07-05-1991
		US 5459047 A	17-10-1995
		US 5166058 A	24-11-1992
		US 5635373 A	03-06-1997
		US 5849880 A	15-12-1998
		US 5187076 A	16-02-1993
		US 5618924 A	08-04-1997
		US 5116738 A	26-05-1992
		US 5366875 A	22-11-1994
		NO 963788 A	17-02-1988
		NO 963789 A	17-02-1988
		US 5106748 A	21-04-1992
		US 5108922 A	28-04-1992
		US 5141905 A	25-08-1992